APPENDIX B

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EFFECT OF MULTIMERIZATION OF HUMAN AND RECOMBINANT VON WILLEBRAND FACTOR ON PLATELET AGGREGATION, BINDING TO COLLAGEN AND BINDING OF COAGULATION FACTOR VIII

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Abstract The smallest circulating von Willebrand factor (vWF) molecule is a dimer composed of two identical subunits containing binding sites for heparin, collagen, platelet glycoproteins and coagulation factor VIII (FVIII). Interdimeric disulfide linking leads to multimers composed of up to 40 dimers. vWF serves as a carrier of FVIII and is required for normal interactions of platelets with the subendothelium of the injured vessel wall. Von Willebrand factor was purified from human plasma cryoprecipitate and fermentation supernatant of recombinant CHO cells by anion exchange chromatography. Heparin affinity chromatography was used to isolate vWF polymers of different degree of multimerization. Analysis of collagen binding and platelet aggregation revealed that these activities increase with increasing degree of multimerization of vWF. Binding of FVIII to vWF was studied by real-time biospecific interaction analysis and surface plasmon technology. The binding data showed that the binding of FVIII is independent of vWF multimerization. Using recombinant FVIII and recombinant vWF, real-time biospecific interaction analysis resulted in a potential stoichiometry of 2 to 2.5 vWF-subunits per bound FVIII molecule. The kinetic analysis of the vWF-FVIII interaction resulted in a binding rate constant of about $3 \times 10^4\ M^{-1} s^{-1}$ and an equilibrium dissociation constant of about $0.4 \times 10^{-9}\ M$.

The adhesive protein von Willebrand factor exists in human plasma as a series of heterogeneous homo-multimers ranging in size from about 450 kDa to more than 10,000 kDa (1-4). The precursor polypeptide produced in endothelial cells, pro-pro-vWF, consists of a 22-residue signal peptide, a 741-residue pro-peptide and the 2050-residue polypeptide found in mature plasma vWF. After removal of

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the signal peptide, the resulting pro-vWF subunits are engaged in a complex biosynthetic process thought to begin with the formation of a primary dimer, containing two pro-vWF subunits, through disulfide bond linkage. Then protomeric units of the multimeric series are assembled into higher order multimers by disulfide bonding of dimers. vWF pro-peptide is cleaved from multimeric vWF before release from intracellular storage sites into circulation (1,3,5,6). Once released, vWF serves a dual purpose in hemostasis. First, it acts as an adhesive protein that serves as a bridge between platelet glycoproteins and the vascular subendothelium. Second, vWF binds coagulation factor VIII (FVIII) and circulates with it as a noncovalently linked complex. In this manner, vWF plays a key role in hemostasis by initiating platelet adhesion at sites of vascular injury and by localizing FVIII to sites where it can participate in the generation of thrombin and the fibrin clot (7). From analysis of structurally abnormal vWF molecules, proteolytically digested vWF polymers, and fragmentation/chemical modification of vWF (8-19) it is accepted that the highest molecular weight vWF multimers exhibit the highest hemostatic efficacy. However, it would be advantageous to isolate native vWF molecules with different degree of multimerization without modification of vWF 's structure, followed by functional characterization, including coagulation factor VIII binding, platelet aggregation and collagen binding.

In this study, vWF was purified from fermentation supernatant of recombinant CHO cells (recombinant vWF, r-vWF) and from human plasma cryoprecipitate (human plasma-derived vWF, hp-vWF) and was separated by heparin affinity chromatography into fractions composed of vWF multimers with different degree of multimerization. Then, r-vWF and hp-vWF preparations were analyzed for ristocetin-induced platelet aggregation, binding to collagen, and binding of coagulation factor VIII using real-time biospecific interaction analysis and surface plasmon resonance technology.

MATERIALS AND METHODS

Materials
Real-time biospecific interaction analysis using surface plasmon resonance technology (SPR) was performed using a BIAcore™ 2000 from Pharmacia Biosensor, Uppsala, Sweden. Sensor chip CM5 and amine coupling kit containing N-hydroxysuccinimide (NHS), N-ethyl-N'-(3-diethylaminopropyl)-carbodiimide (EDC) and ethanolamine hydrochloride were obtained from Pharmacia Biosensor, Uppsala, Sweden. Recombinant von Willebrand factor (r-vWF) produced by large scale fermentation of recombinant CHO cells (20, 21), human plasma, human plasma cryoprecipitate and purified monoclonal anti-von Willebrand factor antibody, MAb AvW8/2, were from IMMUNO, Vienna, Austria. Recombinant coagulation factor VIII (Kogenate™, r-FVIII) was from Bayer, Leverkusen, Germany. Enzyme immunoassay for quantitative determination of vWF (Asserachrom vWF) was from Boehringer Mannheim, Mannheim, Germany. Assay for vWF ristocetin cofactor activity (Von Willebrand Reagent) was from Behringwerke, Germany. Fractogel EMD-TMAE and Fractogel EMD-Heparin were from Merck, Darmstadt, Germany. Rabbit anti-human von Willebrand factor serum was from Dako, Glostrup, Denmark. Alkaline phosphatase conjugated affinity purified goat anti-rabbit IgG was from Bio-Rad, Hercules, USA.

Purification of vWF
Purification of vWF from human plasma cryoprecipitate (hp-vWF) and purification of recombinant vWF (r-vWF) from cell culture supernatant of recombinant CHO cells was performed in 20 mM Tris/HCl buffer, pH 7.4 (Tris-buffer), by combination of anion exchange chromatography and heparin-affinity chromatography. Human plasma cryoprecipitate and

recombinant CHO cell supernatant were applied onto Fractogel EMD-TMAE column equilibrated with Tris-buffer. First, Fractogel EMD-TMAE column was washed with 180 mM NaCl in Tris-buffer. Then, vWF was eluted from Fractogel EMD-TMAE with 280 mM NaCl in Tris-buffer. vWF containing fractions were diluted with Tris-buffer to 90 mM NaCl and were filtered through Fractogel EMD-heparin column. Weakly bound material was eluted with 100 mM NaCl in Tris-buffer. vWF was eluted from Fractogel EMD-heparin column by 120 mM NaCl, 160 mM NaCl, 190 mM NaCl, 230 mM NaCl and 280 mM NaCl in Tris-buffer.

Qualitative and quantitative characterization of vWF

Concentration of vWF antigen (vWF:Ag) was determined by an enzyme immunoassay. vWF:Ag determination was not affected by multimer sizes of vWF (data not shown). Binding activity of vWF to collagen was determined using the method described previously (22). Briefly, human collagen III was coated on microtiter plates. vWF dilutions were then incubated with immobilized collagen. Collagen-bound vWF was quantified using rabbit anti-human vWF IgG-peroxidase conjugate. Ristocetin cofactor activity of vWF (RistoCoF) was determined by a ristocetin cofactor assay. Multimer analysis of vWF was performed by 1 % agarose gel electrophoresis as described previously (20, 21). Briefly, individual vWF multimers were separated by agarose gel electrophoresis and were then blotted onto nitro-cellulose membrane. Visualization of vWF multimers was carried out by immunoenzymatic staining using as primary antibody a rabbit anti-human von Willebrand factor serum. As a secondary antibody alkaline phosphatase conjugated affinity purified goat anti-rabbit IgG was used and staining was performed with the nitroblue tetrazolium chloride / bromochloro-indolyl-phosphate substrate system.

Real-time biospecific interaction analysis

Monoclonal anti-human von Willebrand antibody AvW8/2 was covalently bound to sensor chip CM5 surface by reaction of AvW8/2 associated amines with an N-hydroxysuccinimide-ester activated sensor chip surface (23, 24). Measurement of response units after AvW8/2 immobilization resulted base line response units (RU$_{BL}$). r-vWF and bp-vWF were dissolved in 10 mM HEPES pH, 7.4, 150 mM NaCl, 1 mM CaCl$_2$, 0.05 % surfactant P20 (HBS-buffer) to a vWF:Ag concentration of 20 µg/ml. For each experiment a 50 µl sample of vWF dilution was injected over the sensor chip surface with a constant flow rate of 5 µl/min and was allowed to interact with immobilized AvW8/2 (phase A). For each experiment a constant level of 0.55 ng vWF were bound per mm^2 sensor chip surface. Non-bound vWF was washed from the chip surface by injecting 20 µl HBS-buffer (phase B). bp-vWF was additionally washed with 20 µl 250 mM CaCl$_2$ to elute concomitant traces of plasma coagulation factor VIII. Measurement of response units after binding of vWF to immobilized AvW8/2 resulted in vWF response units (RU$_{vWF}$). 60 µl r-FVIII (2.5 µg/ml in HBS-buffer) were injected over the sensor chip surface at a flow rate of 5 µl/ml to react with vWF bound to AvW8/2 at the chip surface. Binding of r-FVIII to vWF was recorded continuously (phase C). Measurement of response units at the end of the association phase resulted in FVIII response units (RU$_{FVIII}$). The flow was continued at the same rate with HBS-buffer, and dissociation of r-FVIII was followed continuously (phase D). Apparent stoichiometry of the vWF$_{\text{---}}$-FVIII complex was calculated by equation: vWF$_{\text{---}}$: FVIII = (RU$_{vWF}$ - RU$_{BL}$) / (RU$_{FVIII}$ - RU$_{vWF}$) x 330,000 / 225,000, where 330,000 and 225,000 refers to the molecular weights of FVIII and vWF, respectively. Dissociation rate constants (k$_d$) and association rate constants (k$_a$) were calculated as described in detail previously (23, 24) using micro-computer soft-ware provided by Pharmacia Biosensor. Equilibrium dissociation constant (K$_d$) resulted from K$_d$ = k$_d$ / k$_a$. Each binding analysis was performed five times. Coefficient of variation was less than 5% of the mean value.

RESULTS

vWF was purified from human plasma cryoprecipitate and from fermentation supernatant of recombinant CHO cells by combination of anion exchange chromatography and heparin affinity chromatography (Fig. 1). Prior purification, hp-vWF and r-vWF exhibited ristocetin cofactor activities of 27 mU/µg vWF:Ag and 15 mU/µg vWF:Ag, respectively. Elution of anion exchange column with 280 mM NaCl led to vWF preparations with the entire multimer pattern, ranging from the first dimer up to the largest vWF-polymers. Ristocetin cofactor activities of 33 mU/µg vWF:Ag and 15 mU/µg vWF:Ag were obtained for hp-vWF and r-vWF, respectively. By contrast, elution of heparin affinity column by different NaCl concentrations resulted in vWF with various degree of multimerization. Elution of heparin affinity column with 120 mM NaCl yielded vWF composed mainly of vWF-dimer and traces of vWF-tetramer. Elution at 160 mM NaCl resulted in a mixture composed of vWF-dimer, vWF-tetramer and trace amounts of vWF-hexamer. A mixture of vWF-dimer to vWF-octamer was isolated with 190 mM NaCl and a mixture mainly composed of vWF-dimer to vWF-dodecamer was eluted with 230 mM NaCl. A complete range of vWF-polymers, from the dimer up to the largest polymers, was obtained at 280 mM NaCl. Re-chromatography of vWF preparations of specific polymer composition led to

A

B

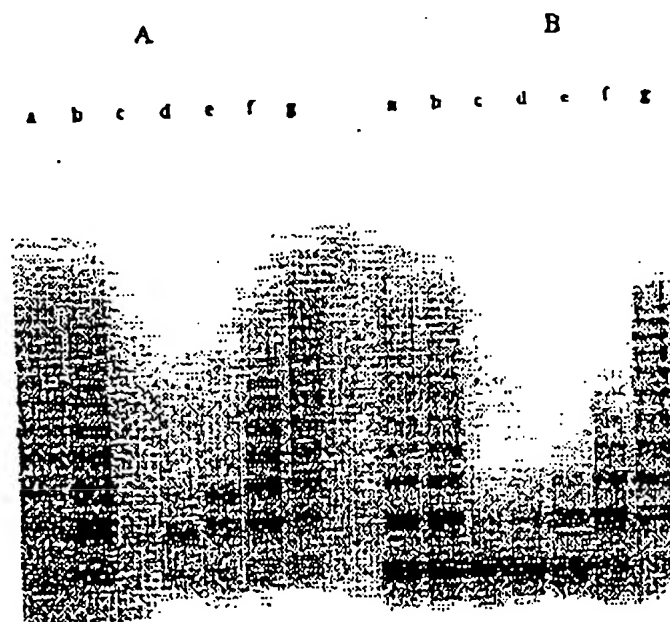a b c d e f g   a b c d e f g

FIG. 1

Purification and separation of hp-vWF (A) and r-vWF (B) with various degree of polymerization. Multimer analysis. (a) vWF before purification; (b) 280 mM NaCl eluate from Fractogel EMD-TMAE; (c) 120 mM NaCl eluate from heparin-affinity chromatography; (d) 160 mM NaCl eluate from heparin-affinity chromatography; (e) 190 mM NaCl eluate from heparin-affinity chromatography; (f) 230 mM NaCl eluate from heparin-affinity chromatography; (g) 280 mM NaCl eluate from heparin-affinity chromatography.

TABLE 1

Heparin-Affinity Chromatography of vWF. Elution of vWF Fractions at Different NaCl Concentrations. Determination of the Ratio of Ristocetin Cofactor Activity to vWF Concentration (RistCoF / vWF:Ag) and the Ratio of Collagen Binding to vWF Concentration (Collagen binding / vWF:Ag).

Sample	RistoCoF/vWF:Ag (mU/µg)	Collagen binding/v WF:Ag (mU/µg)
hp-vWF heparin-affinity eluates at		
120 mM NaCl	0	0
160 mM NaCl	0	0
190 mM NaCl	6.3	1.9
230 mM NaCl	36.0	14.1
280 mM NaCl	76.0	40.0
r-vWF heparin-affinity eluates at		
120 mM NaCl	0.1	0
160 mM NaCl	0.4	0.1
190 mM NaCl	1.0	0.31
230 mM NaCl	7.6	3.0
280 mM NaCl	25	35

no further separation of individual vWF multimers, but elution of vWF at the original NaCl concentration. Separation of vWF-multimers was performed in the absence of calcium ions. By contrast, addition of 10 mM calcium ions resulted in no separation of vWF polymers.

All vWF preparations isolated by heparin affinity chromatography were analyzed for ristocetin cofactor activity, collagen binding and vWF concentration (vWF:Ag). The individual ratio of ristocetin cofactor activity to vWF:Ag and collagen binding activity to vWF:Ag are summarized in Tab. 1. The comparison between the multimer composition of individual vWF preparations and the functional properties shows that both platelet aggregation activity and collagen binding activity of as well as hp-vWF and r-vWF increase with increasing multimerization of vWF. Highest activities were detected in vWF preparations containing high molecular weight multimers.

Interaction of vWF and coagulation factor VIII was analyzed by real-time biospecific interaction analysis and surface plasmon resonance technology (25). The sensor chip surface was activated and the monoclonal anti-human vWF antibody AvW8/2 was covalently immobilized at the chip surface via amine coupling. Measurement of response units after coupling yielded the base line value (RU$_{BL}$). Then, vWF preparations were injected over the sensor chip surface and vWF was captured by immobilized AvW8/2 (phase A). For each binding analysis 0.55 ng vWF:Ag were bound per mm^2 of sensor chip surface. This resulted in an increase in the protein mass bound to the sensor chip surface and in an increase in the response units (RU$_{vWF}$). Non-bound vWF was washed from the surface by buffer (phase B). The difference between RU$_{BL}$ and RU$_{vWF}$ is a measure for vWF concentration bound to the sensor chip surface. Next, r-FVIII was injected over the sensor chip surface. Binding of r-FVIII to captured vWF resulted in a further increase in

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TABLE 2

Biospecific Interaction Analysis of vWF and FVIII. Determination of Rate Constants and Equilibrium Dissociation Constants.

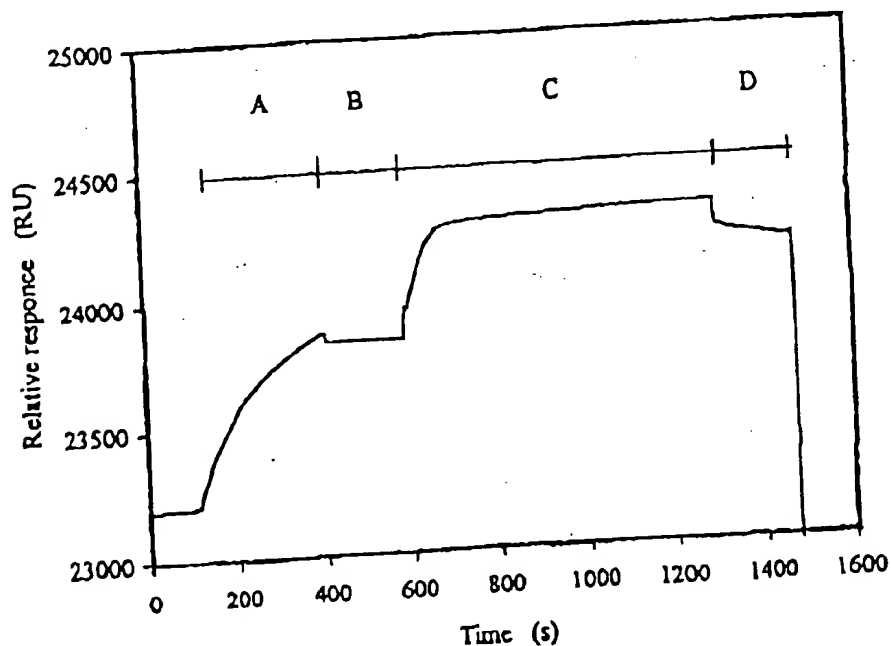| Sample	Stoichiometry vWF-subunit : FVIII	k_a (M⁻¹ s⁻¹) $\times 10^6$	k_d (s⁻¹) $\times 10^{-3}$	K_d (M) $\times 10^{-9}$
r-vWF 120 mM eluate	2.25 : 1	4.53	1.85	0.41
r-vWF 160 mM eluate	2.5 : 1	3.9	1.8	0.46
r-vWF 190 mM eluate	2.0 : 1	3.75	1.66	0.44
r-vWF 230 mM eluate	2.0 : 1	3.19	1.38	0.43
r-vWF 280 mM eluate	2.0 : 1	3.0	1.16	0.39

FIG. 2

Kinetic analysis of the interaction of r-vWF and r-FVIII. 50 µl r-vWF (280 mM NaCl eluate from heparin-affinity chromatography, Fig.1B-g) at a concentration of 20 µg/ml was injected over the sensor chip surface (phase A) to be captured by the MAb AvW8/2. Non-bound r-vWF was eluted from the chip surface by HBS-buffer (Phase B). 60 µl r-FVIII (2.5 µg/ml) were injected over the sensor chip surface to bind to the captured r-vWF (phase C). r-FVIII was dissociated from the r-vWF-r-FVIII complex by washing the sensor chip surface with HBS-buffer (phase D).

response units (phase C). Flow of r-FVIII was continued until saturation of vWF was reached. Response unit measurement at this time yielded RU_{FVIII} value. The difference between RU_{FVIII} and RU_{vWF} corresponds to the amount of r-FVIII bound to r-vWF. HBS-buffer was injected over the sensor chip surface to allow dissociation of r-FVIII from r-vWF (phase D). A typical sensorgram is shown in Fig. 2. To study r-FVIII binding, r-vWF preparations varying in multimer compositions, as well as hp-vWF were applied. Apparent stoichiometry of the complex of $vWF_{subunit}$ to FVIII molecule, apparent association rate constants (k_a), apparent dissociation rate constants (k_d) and equilibrium dissociation constants (K_d) were determined. Data are summarized in Tab. 2. Injection of r-FVIII at concentrations of 1 μg/ml and 5 μg/ml resulted similar binding data (not shown).

DISCUSSION

The vWF molecule contains two different heparin binding domains. A calcium-dependent heparin binding domain is located on the N-terminal fragment of vWF subunit (amino acid 1-298) and a second heparin binding domain is located in the A1 domain, presumable in the loop region between Cys_{509} and Cys_{695} (1, 3, 13, 15-17, 26, 27). Our investigations showed that heparin affinity chromatography of as well as r-vWF and hp-vWF in the presence of calcium ions resulted in elution of all the different vWF-multimers at about 300 mM NaCl concentration. By contrast, calcium-free heparin affinity chromatography led to size-dependent elution of vWF polymers. Contrary to our first working hypothesis to separate distinct vWF fractions of increasing degree of multimerization by increasing the salt concentration during the heparin affinity chromatography, vWF-multimers of increasing degree of polymerization were always accompanied by a set of vWF-multimers of lower degree of multimerization.

vWF acts as a bridge between components of the vessel wall, e.g. collagen, and specific receptors on the platelet surface. Platelets have two distinct receptors for vWF: the glycoprotein Ib (GP Ib) in the glycoprotein Ib/V/IX complex and the activated glycoprotein IIb-IIIa complex (1). Binding of vWF to collagen can easily mimicked in vitro by its binding to collagen surfaces in vWF- collagen ELISA assays. Platelet agglutination is usually studied in vitro in the presence of ristocetin, resulting ristocetin cofactor activity of vWF. Binding sites of vWF to collagen and to platelet receptors are localized in the A1 and A3 domain of vWF (1, 13-16, 18, 19, 28-32). The results obtained for hp-vWF and r-vWF molecules showed that both collagen binding activity and platelet agglutination activity increase with the degree of multimerization of vWF. Apparently, vWF-dimer and vWF-tetramer exhibited collagen binding and platelet aggregation activities below detection limits, while highest activities were measured for high molecular weight vWF-polymers.

Studies by Mannucci et al. (8) have shown that the preparation of cryoprecipitate from human plasma results in an increased degradation of vWF-polymers during the industrial manufacturing process of isolation of vWF and FVIII. Unfortunately, this is accompanied by the formation of low molecular weight vWF and a loss of vWF and FVIII activity. Degradation of vWF in the cryoprecipitate is confirmed in Fig. 1A. Prior heparin affinity chromatography, hp-vWF exhibited a specific ristocetin cofactor activity of 33 mU/μg vWF:Ag. Due to the separation of hp-vWF degradation products and low molecular weight hp-vWF multimers, purified high molecular weight hp-vWF multimer exhibited a platelet aggregation activity similar to vWF isolated from human plasma. During purification of hp-vWF by heparin affinity chromatography, the specific collagen binding activity increased from 17 mU/μg vWF:Ag to 40 mU/μg vWF:Ag. due to the removal of low molecular weight vWF multimers and vWF degradation products.

The second function of vWF is the binding of coagulation factor VIII (FVIII). vWF and FVIII circulate in plasma as a noncovalently linked complex. A specific binding domain for FVIII is localized within amino acids 1 to 272 of vWF. Factor VIII binding to vWF has been investigated using various techniques, such as coating vWF directly on plastics (47, 48), binding vWF with a MoAb (49, 50) and gel filtration (51). Human and porcine vWF and FVIII preparations have been used resulting dissociation constants of 0.38×10^{-9} M to 0.62×10^{-10} M (47, 50, 52). In addition of impurities of FVIII in plasma-derived vWF and vWF-impurities in FVIII preparations used for these studies, binding of vWF to microtitration plates and gold particles may alter its binding function. Quantification of vWF-bound FVIII by antibody-conjugates or by FVIII activity measurements may reflect only a specific proportion and specific properties of the vWF-FVIII complex.

In the present investigation real-time biospecific interaction analysis has been used for the first time to study the interactions between recombinant FVIII and recombinant vWF. In this technology, vWF molecules are bound to a hydrophilic dextran gel and mass concentrations of biomolecules are measured by surface plasmon resonance technology at the real time scale. Binding rate constants, equilibrium dissociation constants and stoichiometry of vWF-monomer/FVIII complex at saturation were calculated. At saturation of vWF with FVIII, a stoichiometry of 2 to 2.5 r-vWF-subunits per r-FVIII molecules was obtained, independent of the degree of vWF polymerization. This result corresponds well with gel filtration experiments by Vlot et al. (52), who found 0.5 factor VIII molecules per vWF monomer. By contrast, after immobilization of vWF at a solid surface (52) vWF was saturated by 23 times less factor VIII, reflecting changed binding and/or steric properties of immobilized vWF. Measurement of the vWF-FVIII interaction at a porous hydrophilic dextran gel in this study indicates that about half of FVIII-binding sites are accessible in the vWF multimer. Purified high molecular weight hp-vWF resulted a stoichiometry of 2.6 hp-vWF-subunits per FVIII molecule. Slightly reduced binding capacity of hp-vWF may result from the proteolytic degradation of plasma-derived vWF, which is missing in r-vWF (20, 21). Apparently, both association rate constants and dissociation rate constants are slightly affected by the degree of multimerization of vWF. Dissociation rate constant of 1.16×10^{-3} s^{-1} determined for high molecular weight r-vWF-multimer and r-FVIII agrees well with the dissociation rate constant of 5.4×10^{-2} min^{-1} of hp-vWF and FVIII determined by Leyte et al. (50). In addition, our results show that the equilibrium binding constants are independent of the degree of multimerization of vWF.

Plasma concentrates containing vWF are used in the therapy of patients with von Willebrand disease unresponsive to the nontransfusional agent desmopressin. However, therapeutic plasma concentrates containing vWF (i) lacking in the largest multimers, and (ii) exhibiting various degree of degradation of vWF (8). Apparently, the structural damage to plasma-derived vWF is caused by soluble and platelets/leukocytes associated proteases and occurs at early stages of the manufacturing process of plasma concentrates. Thus, calcium-free heparin affinity chromatography substantially improves the quality of vWF isolated from plasma cryoprecipitate.

An other way of preparation of von Willebrand factor without the risk of degradation of the molecule by plasma proteases is its recombinant production. Von Willebrand factor and various fragments have been expressed at laboratory scale as well as in prokaryotic and animal cells (13, 33-45). However, although multimerization of r-vWF has been reported in most of these studies, quantitative data of ristocetin cofactor activity and of collagen binding activity, based on activity levels of hp-vWF, have not been published. This may be due to abnormal high proportion of r-vWF dimer and low molecular weight multimers visible in these recombinant vWF preparations

(13, 32, 33, 35, 36, 38, 40, 42, 44, 46) resulting in only low ristocetin cofactor activities. Our previous structural analysis (20, 21) have shown that r-vWF is not degraded by proteases during the fermentation and purification process and that r-vWF is composed of mature homo-dimers. The present results show that r-vWF produced under serum-free fermentation conditions exhibits all qualitative and quantitative functional properties which allow it to mediate platelet aggregation, promote collagen binding and binding of coagulation factor VIII with activity efficiency comparable to human plasma-derived vWF.

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